Supplemental Data

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Virus-Induced Unfolded Protein Response Attenuates Antiviral Defenses via Phosphorylation-Dependent Degradation of the Type I Interferon Receptor

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Supplemental Figures:

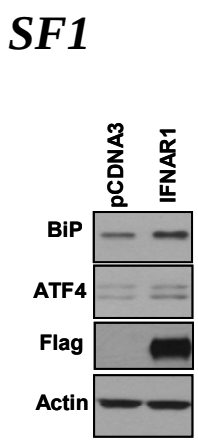

Figure 1. 293T cells were transfected with Flag-IFNAR1 or empty vector (pCDNA3) and the lysates were analyzed by immunoblotting using the indicated antibodies.

SF2

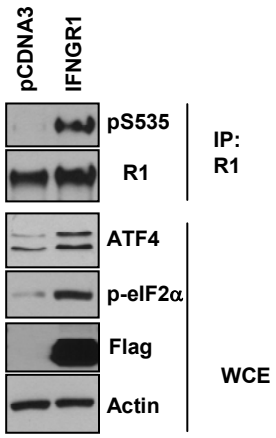

Figure 2. 2fTGH cells were transfected with Flag-IFNGR1 or empty vector (pCDNA3) and whole cell lysates (WCE) were prepared. Endogenous IFNAR1 was immunoprecipitated and analyzed by IB using anti-pS535 and anti-IFNAR1 (R1) antibodies. WCE were analyzed by immunoblotting using the indicated antibodies.

SF3

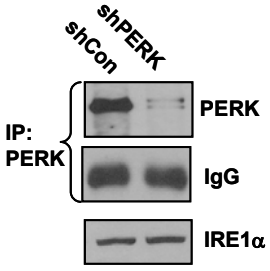

Figure 3. Characterization of shRNA against human PERK. 293T cells were transfected with control shRNA plasmid or shPERK plasmid. 48 h after transfection, cells were harvested and the cells lysates were subjected to analysis for PERK levels by IP-IB. Levels of IRE1 in the lysates serve as loading control.

SF4

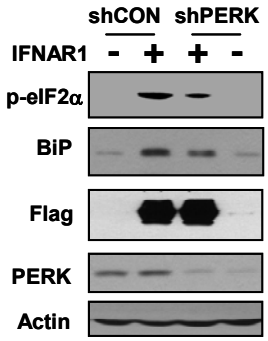

Figure 4. 293T cells were co-transfected with Flag-IFNAR1 along with control shRNA plasmid or shPERK plasmid as indicated. 48 h after transfection, cells were harvested and the cells lysates were subjected to immunoblotting analysis using the indicated antibodies.

SF5

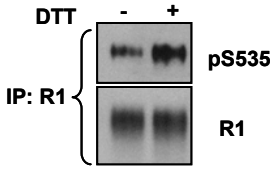

Figure 5. 293T cells were pre-treated with methylamine (20mM) for 1 h and then with 5mM of DTT for 30 min. Lysates were subjected to IP-IB analysis for pS535 and total IFNAR1 levels.

SF6

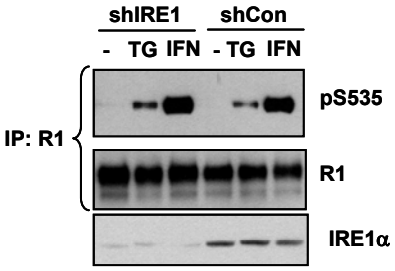

Figure 6. 293T cells were transduced with lentiviruses encoding control shRNA (shCon) or shIRE1α (shIRE1). The cells were treated with TG (1μM) or IFNα (1000 IU/ml) for 30 min. pS535 and total IFNAR1 levels were examined using IP-IB. The effect of IRE1α knockdown was also determined by direct immunoblot.

SF7

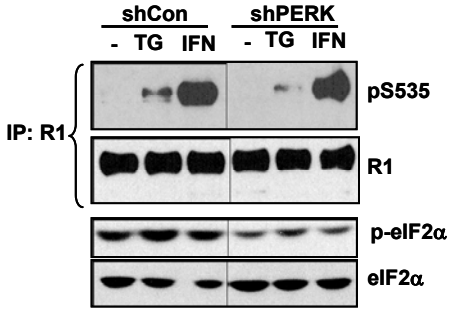

Figure 7. 293T cells transfected with a control shRNA construct or shRNA against PERK were treated with TG (1μM) or IFNα (1000 IU/ml) for 30 min. Phosphorylation and levels of endogenous IFNAR1 and eIF2α were analyzed by immunoblotting.

SF8

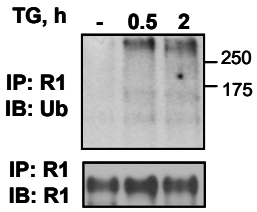

Figure 8. 293T cells pre-treated with methylamine HCl (MA, 20mM) for 1 h were then treated with TG (1μM) for indicated time. Ubiquitination of endogenous IFNAR1 was analyzed by IP-IB using the indicated antibodies.

SF9

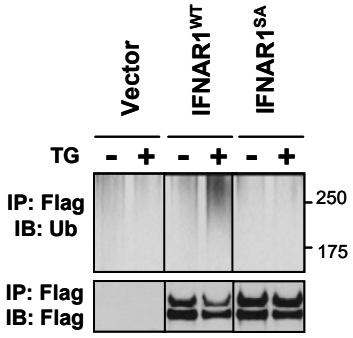

Figure 9. MEF cells derived from IFNAR1-null mice were stably transduced with either empty retrovirus ("Vector) or with retroviruses for expression of mouse Flag-tagged IFNAR1 (wild type or S526A mutant, "SA"). Effect of TG treatment on IFNAR1 ubiquitination was analyzed by IP-IB using the indicated antibodies.

SF10

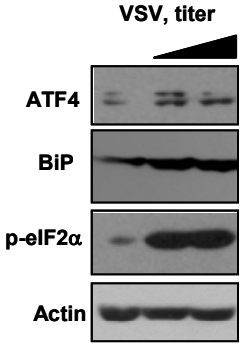

Figure 10. 2fTGH cells were left untreated or infected with VSV (MOI 0.1 and 0.3 respectively) for 19 h. Levels of indicated proteins were analyzed by immunoblotting using indicated antibodies.

SF11

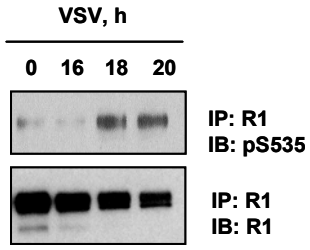

Figure 11. 2fTGH cells were infected with MOI 0.1 VSV for 16, 18 and 20 h. pS535 and total IFNAR1 levels were determined.

SF12

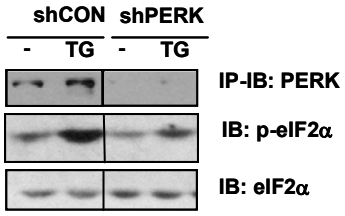

Figure 12. 2fTGH cells transduced with control virus (shCON) or virus encoding shPERK (shPERK) were treated with TG (1 μM) for 30 min. PERK levels were determined by IP-IB. Levels of p-eIF2α or total eIF2α were determined by direct immunoblot.

SF13

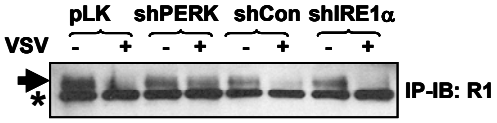

Figure 13. 2fTGH cells transduced with empty virus (pLK), virus encoding shPERK (shPERK), irrelevant control shRNA (shCon) or shIRE1 were infected with MOI 0.1 of VSV for 20 h. Total IFNAR1 levels were determined by IP-IB. Position of mature IFNAR1 is indicated by arrow. Asterisks points to a non-specific band that serves as loading control.

SF14

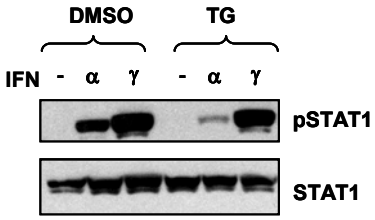

Figure 14. 293T cells were treated with TG (1μM) for 4 h and then the cells were re-fed with fresh medium overnight. After that incubation, cells were treated with 50 IU/ml of IFNα or IFNγ for 30 min. pSTAT1 and STAT1 levels were determined by immunoblotting.

SF15

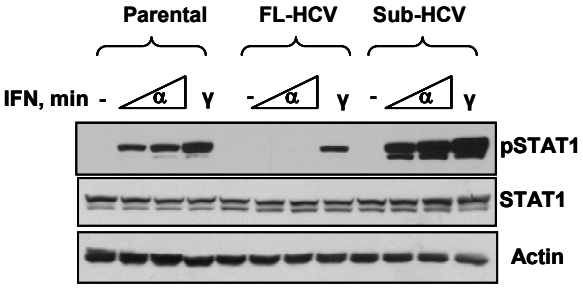

Figure 15. Parental Huh7 cells or cells harboring the subgenomic (Sub-HCV) or the full length (FL-HCV) HCV genome were treated with 50 IU/ml of IFNα (0.5 h and 1 h) or IFNγ (0.5 h). pSTAT1, STAT1 and β-actin levels were analyzed.

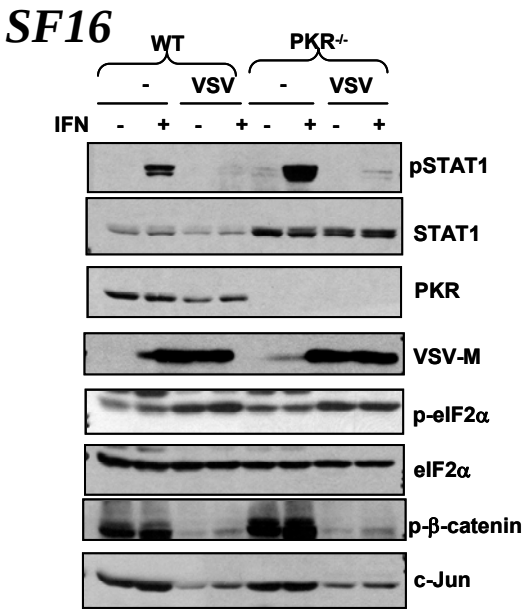

Figure 16. WT or PKR$^{-/-}$ MEFs were infected with MOI 0.1 of VSV for 20 h. Cells were then treated with mIFNβ (50 IU/ml) for 30 min. pSTAT1 and total STAT1 levels were determined. VSV-M, p-eIF2α and levels of two short-lived proteins p-β-catenin and c-Jun were used to show similar levels of viral and stress load in either cell line. Total eIF2α levels were used as loading control.

SF17

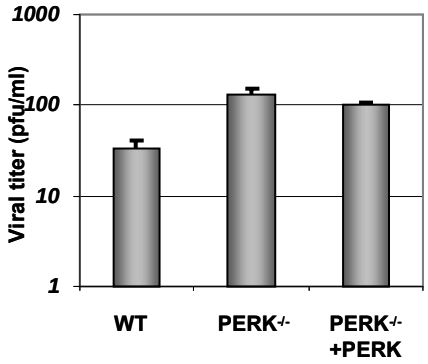

Figure 17. WT or conventional PERK-/- MEFs or these MEFs stably expressing exogenous PERK were infected with VSV. Twenty hours later, virus-containing culture supernatant was harvested and viral titer was determined. Levels of PERK expression are shown in SF18.

SF18

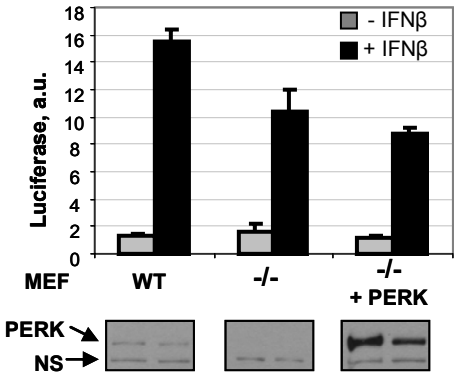

Figure 18. WT or conventional PERK-/- MEFs or these MEFs stably expressing exogenous PERK were transfected with ISRE-luciferase reporter along with Renilla luciferase reporter plasmid. IFNβ-induced ISRE-driven transcription was analyzed as previously described (Kumar et al., 2003). Average activity (in arbitrary units normalized per renilla luciferase) from three independent experiments is shown. Lower panel depicts IB analysis of the lysates from these cells using anti-PERK antibody. Position of PERK is indicated by an arrow. A non-specific (NS) band serves as a loading control.

SF19

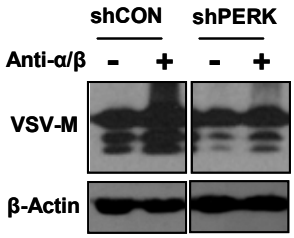

Figure 19. 2fTGH cells harboring either shPERK or irrelevant control shRNA (shCon) were infected with MOI 0.1 of VSV. Blocking antibodies against IFNα and IFNβ (at 500U) were added to the medium at that time as indicated. Cells were harvested 16 h later and the levels of VSV-M and β-actin were analyzed by IB.

Experimental Procedures

Plasmids and Reagents

Vectors for bacterial expression of GST-ctIFNAR1 and mammalian expression of human and mouse Flag-IFNAR1 were described previously (Kumar et al., 2004; Kumar et al., 2007; Kumar et al., 2003); other plasmids were generous gifts from J. Darnell (Flag-STAT1), R. Bartenschlager (HCV constructs) and K.U. Wagner (Cre). All shRNA constructs used were based on pLKO.1. The specific hairpin sequences are outlined in. Recombinant human IFNα2 was purchased from Roche Diagnostics. Recombinant human and mouse IFNγ and mouse IFNβ were purchased from PBL. Thapsigargin, cycloheximide and methylamine HCl were purchased from Sigma.

Cell culture and Virus

All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (Hyclone) and various selection antibiotics when indicated. 2fTGH and its IFNAR2-deficient derivative line U5A were generous gifts of G. R. Stark. 11,1-derivative cell lines that contain WT or kinase-dead form of Tyk2 were a gift from S. Pellegrini (Marijanovic et al., 2006) and were cultured in the presence of 400 μg/ml of G418. PERK-deficient MEFs and its WT counterparts were generous gifts from David Ron (NYU School of Medicine). To acutely delete PERK in MEFs, MEFs harboring $PERK^{fl/fl}$ were infected with control retrovirus or retrovirus expressing Cre. The transduced cells were selected by puromycin for 72 h. The surviving clones were pooled and used for further analysis. IFNAR1-null MEFs reconstituted with pBABE-puro-based retroviral vector encoding Flag-tagged

mIFNAR1^{wt} and mIFNAR1^{S526A} (Kumar et al., 2003) were generated and cultured in the presence of 4 μg/ml of puromycin.. Huh7-derivative cells introduced with a complete HCV genome or a subgenomic genome were described in details in (Luquin et al., 2007) and were cultured in the presence of 500μg/ml of G418.

Mouse ES clone harboring a S526A mutation were obtained by homologous recombination. The targeting vector containing this mutation (Figure 3A) was introduced via electroporation into the C57/BL6 ES cells. The cells were subjected to neomycin selection and DNA samples from survived clones were analyzed by Southern blotting using the indicated probes to identify the homologous recombinants. For experiments, ES cells were differentiated into embryonic bodies according to ATCC recommendations established protocol (Maatman et al., 2003). The embryonic bodies were trypsinized and plated in gelatinized plates using IMDM containing 10% FBS. VSV (Indiana serotype, a gift from R. Harty) was propagated in HeLa cells.

Transfections and lentiviral vector-mediated gene knockdown

Transfection of 293T cells and KR-2 cells using LIPOfectamine Plus and of Huh7-derivatives using LIPOfecatimine-2000 (Invitrogen) was carried out according to manufacturer's recommendations. Replication-deficient lentiviral particles encoding shRNA against GFP (shCON), hPERK and hIRE1α, or the empty virus control were prepared via co-transfecting 293T cells with three other helper vectors as previously described (Dull et al., 1998). Viral supernatant were concentrated by PEG8000 precipitation and were used to infect 2fTGH and U5A lines in the presence of 3μg/ml of polybrene (Sigma). Cells were selected and maintained in the presence of 1.5 μg/ml of puromycin.

Cell treatment and viral infection

For examining the signaling event occurring after initiation of ER stress, cells were treated with vehicle (DMSO) or TG (1μM, unless otherwise indicated) for 0.5 – 2 h as shown in the figure legends. Unless otherwise specified, cells were inoculated with VSV at an initial MOI of 0.1-1.0 for 1 h. After removing the virus inoculum, cells were then fed with fresh medium. Cells were harvested at different time points afterwards; most of the effects were observed when the cells were uniformly infected and viral markers were at saturation. In some experiments, virus-infected cells were pulsed with IFNs for 30 min and then harvested.

To examine the anti-viral effect of IFN in relation to the time of its addition, 20 IU/ml of IFN was either added overnight prior to VSV infection or was added after the initial virus inoculation/removal. Culture supernatant was generally harvested 20 h after the initial inoculation for analysis of viral titer. VSV titer determination was performed as described elsewhere (Sharma et al., 2003). Briefly, viral supernatant was harvested from triplicate wells and was serially diluted with DMEM+10% FBS. The diluted viral medium was fed to confluent HeLa layer in 12-well plates. 1 h after inoculation, the supernatant was removed and the cell layer was washed twice with PBS. The cells were then overlaid with 1.5 ml of semi-solid medium (DMEM containing 5% FBS and 1% methyl-cellulose) and cultured for an additional 24 h. The cells were then fixed and stained with 0.1% crystal violet. The plaques were counted and presented as plaque-forming units (pfu)/ml.

Immunotechniques

Antibodies against pSTAT1, p-eIF2α, p-β-catenin, β-catenin, IRE1α (Cell Signaling), STAT1 (Cell Signaling), eIF2α (Biosources), hIFNAR1, PKR, c-Jun, IκBα (Santa Cruz), mIFNAR1 (R&D Systems), Flag tag, β-actin (Sigma) and ubiquitin (clone FK2, Biomol), ISG15 and PERK (Rockland) were used for immunoprecipitation and immunoblotting. Monoclonal antibody 23H12, specific for the M protein of VSV (VSV-M), was kindly provided by D. S. Lyles (Wake Forest University School of Medicine, Winston-Salem, N.C.). Antibody against IFNAR1 phosphorylated on Ser535 (in human receptor) or Ser526 (in murine receptor) were described previously (Kumar et al., 2004). Cells lysis, immunoprecipitation and immunoblotting procedures were described earlier (Kumar et al., 2004). Kinase assay with cell lysates and GST-ctIFNAR1 as a substrate was previously described (Liu et al., 2008).

Additional Details for Experimental Procedures:

Plasmid and viruses:

shCon (CAACAAGATGAAGAGCACCAA), shIRE1α (GAGAAGATGATTGCGATGGAT) and shPERK (CCTCAAGCCATCCAACATATT plasmids based on pLKO.1-puro vector (Sigma) were used in either transient transfection experiments or were used to generate lentiviruses encoding the short hairpin sequences to infect 293T or 2fTGH cells.

Cells and Transfections:

PERK-deficient MEFs and its WT counterparts were generous gifts from David Ron (New York University). $PKR^{-/-}$ MEFs and their WT counterparts were geneous gifts of R. Kaufman (University of Michigan). 293T cells were transfected with shRNA plasmids using LIPOfectamine Plus reagent (Invitrogen) according to manufacture's instructions. Studies on HCV were carried out using HCV genomic and subgenomic replicon system. Stable derivatives of Huh7 human hepatic cell line that express either incomplete genome of HCV (lacking structural proteins) or complete HCV genome (that expresses structural proteins as well) were generated and characterized as previously described (Luquin et al., 2007).

In vitro kinase assay:

In vitro PERK kinase assay using GST-cIFNAR1 as a substrate was performed using kinase buffer containing 20 mM HEPES 7.4, 50 mM KCl, 2 mM MgOAC, 2 mM $MnCl_2$, 20 μM ATP and 1.5 mM DTT. Recombinant $PERK^{\Delta N}$ and GST-IFNAR1 were described previously (Cullinan et al., 2003; Kumar et al., 2003). 5 ng of $PERK^{\Delta N}$ and 1 μg of GST-IFNAR1 was mixed in the kinase buffer containing 1 μCi of γ-ATP. The reaction mix was incubated at 30°C for 30 min. The samples were separated on SDS-PAGE and analyzed by auto-radiography.

Virus-mediated shRNA knockdown:

Virus packaging was done in 293T cells as described elsewhere (Dull et al., 1998).. Target cells were infected with concentrated virus in the presence of 3 μg/ml of

polybrene. 48 h after transduction, 2fTGH and 293T cells were selected in medium containing 1.5 and 3 μg/ml of puromycin, respectively.

Antibodies:

Antibodies against pSTAT1, p-eIF2α, p-β-catenin, IRE1α (Cell Signaling), STAT1 (Cell Signaling), eIF2α (Biosources), hIFNAR1, PKR, c-Jun (Santa Cruz), β-actin (Sigma) were used in immuno-precipitation or immunoblotting experiments. Anti-pS535 was previously described (Kumar et al., 2004). Anti-serum against PERK was generated using recombinant PERK. Monoclonal antibody 23H12, specific for the M protein of VSV (VSV-M), was kindly provided by D. S. Lyles (Wake Forest University). Blocking antibodies against human IFNα and IFNβ were purchased (PBL).

FACS analysis:

Measurements of surface levels of IFNAR1 in MEFs of various genetic background was carried out using an anti-mIFNAR1 antibody (R&D Services) as previously described elsewhere (Sheehan et al., 2006).

Viral titer determination:

MEFs were infected with an apparent MOI0.1 of VSV for 1 h before the initial inoculum was removed and the cell layer was fed with medium after washed once with PBS. 20 h after infection, the virus-containing culture supernatant was harvested and the viral titer is determined according to previously published report (Sharma et al., 2003). At the time of

harvesting cells for biochemical analyses, cells were infected almost uniformly judging by saturation in the levels of viral markers.

Supplemental References

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